



The Controversial Effect of Vitamin C Combined with Traditional Oncology Therapy

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Accepted on: 30-01-2016; Finalized on: 31-03-2016.

ABSTRACT

Cancer is one of the leading cause of death in the world. Therefore, the interest in the development of new therapeutic strategies based on the potential anticancer activity of natural antioxidants increased in recent years. Complementary therapy based on nutritional therapy has demonstrated the ability to minimize damage to normal tissue due to oxidative stress deriving from traditional chemotherapy or radiotherapy. One possible medical strategy is the use of vitamin C as adjunctive therapy in the treatment of cancer. The studies in recent years inform about familiar antioxidant, potential cardioprotective effect of vitamin C, but also about prooxidant- anticancer action of ascorbate. In combination with doxorubicin, as the most effective chemotherapeutic agent in the treatment of advanced breast cancer, vitamin C has demonstrated a potential synergic antiproliferative effect on MCF-7 cells. This study showing possible antitumor activity of this combination on breast cancer cells by real time cell analysis (RTCA). MCF-7 cells were exposed to (10 μ M - 700 μ M) vitamin C together with 1 μ M doxorubicin. The below concentration at 10 μ M vitamin C with 1 μ M doxorubicin caused familiar antioxidant effect of ascorbate. The cancer cells growth inhibition have been shown combination of 100 μ M vitamin C with 1 μ M doxorubicin. The increasing concentrations of vitamin C (700 μ M) with 1 μ M doxorubicin represent a higher cytotoxic effect on a breast cancer cell line, while IC₅₀ value of vitamin C was 493.

Keywords: Ascorbic acid, doxorubicin, breast cancer, combination therapy, anticancer activity.

INTRODUCTION

Cancer is one of the major public health problems of the world. Among the different types of cancer, breast cancer is one of the most frequently diagnosed. It is the leading cause of cancer death in females around the world.¹ The current management of breast cancer medication reflects a balance between the treatment effect and its toxicity and contain surgery, chemotherapy and radiotherapy.^{2,3} A serious obstacle today's therapy is the presence of subclinical micrometastases with a tendency to grow into clinically relevant macrometastases with survival median in the range of 2-3 years.⁴ Last but not least, chemotherapeutic resistance is becoming more common, during which chemoresistant tumor cells become resistant to drug-induced apoptosis due to changes in the caspase cascade at 3rd level caspase and p53 gene mutation.⁵

However, research has shown useful potential use of combined therapy, including the association between classical cytotoxic and nutritional therapy which supported the antitumour activity.³ One possible medical strategy combination therapy is the use of vitamin C with doxorubicin. Doxorubicin is a potent broad-spectrum antineoplastic antibiotic (produced by *Streptomyces peucetius* var. *Caesius*) belonging to the anthracycline family.^{6,7} Their therapeutic activity results from its intercalating into DNA, thereby inhibiting topoisomerase II and preventing DNA and RNA synthesis.⁸ A generally

accepted safe maximum cumulative dose of doxorubicin is 450 to 500 mg/m² of body surface.⁹

Combination with vitamin C seems to be of particular interest. As ascorbic acid has been found to potential reduce anthracycline induced cardiotoxicity which can compromise the clinical use of doxorubicin.¹⁰ The nowadays results also suggested that ascorbate shown to exhibit selective toxicity malignant against a variety of cell lines, induce cell cycle arrest at the appropriate doses, slows down tumor growth and prolongs survival time in terminal human cancer patients.^{11,12}

Unfortunately, the efficacy vitamin C is still a matter of controversy, as for many other unconventional anticancer agents. Despite an extensive literature exists dealing with the action of vitamin C, the arise different parameters usually defined in these studies (e.g. doses, routes of administration, adverse effects in cancer patients and influence of ascorbate on the phase to oxidative stress).¹³ Ascorbate can act either as a prooxidant or an antioxidant agent depending on the redox status of the cell and its environment, as well as the concentration of ascorbate at the specific time.¹⁴ The prooxidant activity of ascorbate is quite surprising given that this compound is generally considered an antioxidant.¹³

Therefore, the present study was designed to evaluate the potential antineoplastic effects of vitamin C in association with doxorubicin to treat breast cancer.



MATERIALS AND METHODS

MCF-7 cultivation

The Breast adenocarcinoma line of MCF-7 was obtained from the American Type Culture Collection (the United States) in frozen state. Then cryotube (about 8×10^5 cells of MCF-7) was rapidly heated in a water bath (37°C). Thus thawed cell culture was transferred to the 75 cm^2 culture flask (Becton, Dickinson and Company, the United States), which contain 10 ml of culture medium. DMEM (4,5 g/l Glucose, Lonza- BioWhittaker, Belgium) and F-12 HAM medium (Sigma, USA) in ratio 1:1 were supplemented with 5% FBS (GIBCO, USA) and 1% Sodium Pyruvate 100mM (GIBCO, USA). The cell line was incubated at 37°C and 5% CO_2 . The culture medium was changed every 3 days and the 90% confluence were cells passaged and trypsinized by 0.05% trypsin- EDTA (GIBCO, USA). They were subsequently centrifuged for 2 min, at 1800g, and 4°C and transferred to 9 culture flasks (75 cm^2) and after reaching 80% confluence was performed experiment.

Viability of MCF-7 cells

Cell count and viability was measured with the Muse Cell Analyzer (Millipore, Hayward, CA, USA) using the Muse Count and Viability Kit (Millipore, Hayward, CA, USA) which differentially stains viable and dead cells based on their permeability to two DNA binding dyes. Data were presented as proportional viability (%) by comparing the treated group with the untreated cells, the viability of which is assumed to be 100%. Original volume of the cells was 20 ml and we used dilution factor 20: 1×10^6 to 1×10^7 cells/ml. Then we added to 20 μl of cell suspension 380 μl of Muse TM Count et Viability reagent (kit) and incubated for 5 minutes at room temperature.

Cell Proliferation and Cytotoxicity Measurement Using Real-Time Cell-Based Assay

The real-time cell-based assay (RTCA) xCELLigence (Roche Applied Science and ACEA Biosciences, San Diego, CA, USA) consists of four main components: the RTCA analyzer, the RTCA station, the RTCA computer with integrated software and disposable E-plate 16. In 16-well E-plates were pipetted 80 μl culture medium. The plate was equilibrated with the fluid for 30 minutes in a levelling of contact between the medium and the electrodes. The plate with medium has been inserted in the measuring device (housed in a humidified incubator at 37°C with a 5% CO_2 atmosphere) to measure the RTCA xCelligence impedance values background. Then, MCF-7 cells were seeded in E-plates at a density of 20000 cells per well. The MCF-7 cells were monitored using the xCELLigence DP system at 15-min intervals. After 24 h and 15 min. of RTCA profiling, the assay was paused, and the E-Plate 16 was removed from the xCELLigence system. The existing media was carefully removed and the MCF-7 cells were washed with PBS to remove unattached cells. Subsequently 1 μM doxorubicin and different concentration of vitamin C (10 μM - 700 μM) with culture

medium were added. The E-Plate 16 was placed back into the xCELLigence system and MCF-7 cells were monitored for 76 h. Each sample extract was analyzed in duplicate. The results are expressed as relative impedance using the manufacturer's software (Roche Applied Science and ACEA Biosciences). Half-maximal concentration was calculated in the RTCA proprietary software using a sigmoidal dose-response formula and by fitting an area under curve in a time period vs. concentration curve type. Slope and doubling time calculations were performed with RTCA software 1.2 (Roche).

RESULTS

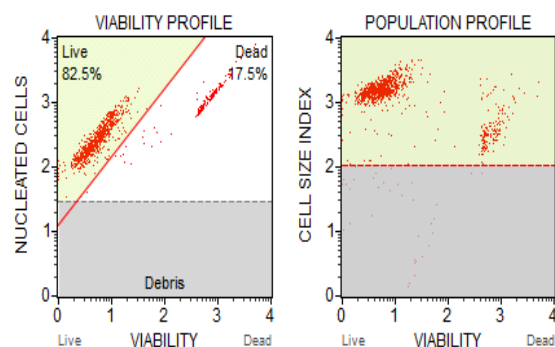


Figure 1: Determination of the number of MCF-7 cells and cell viability using Cell Muse TM (viable cells/mL: $1.13\text{E}+06$ and total cells/mL: $1.37\text{E}+06$).

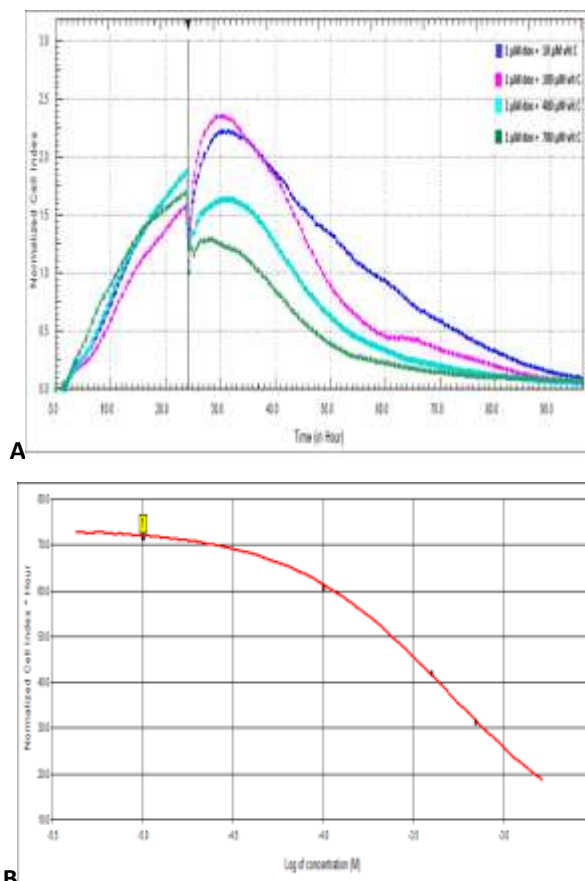


Figure 2: Real-time monitoring of cytotoxic effect of vitamin C on MCF-7 cells. DRC (area-under-curve in a time period vs concentration) Representative IC50 curve for

vitamin C in MCF-7 cells. Chosen time point: 24:15:06 ~ 90:19:43 (Fig. 2A). The IC_{50} value of vitamin C was 493 μ M during 76-h treatment in MCF-7 cells (Fig. 2B).

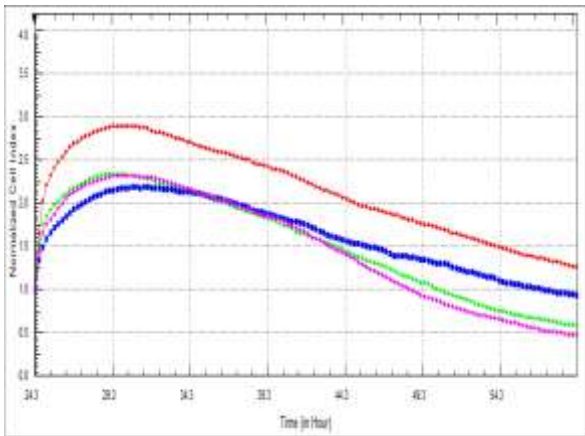


Figure 3: Normalized cell index values in time range: 24:15:06 ~ 59:02:34 during culturing MCF-7 cell line after the addition of cytotoxic drugs; red line – MCF-7 cells, green line – MCF-7 (1 μ M doxorubicin), blue line – MCF-7 (1 μ M doxorubicin + 10 μ M vitamin C), pink line - MCF-7 (1 μ M doxorubicin + 100 μ M vitamin C).

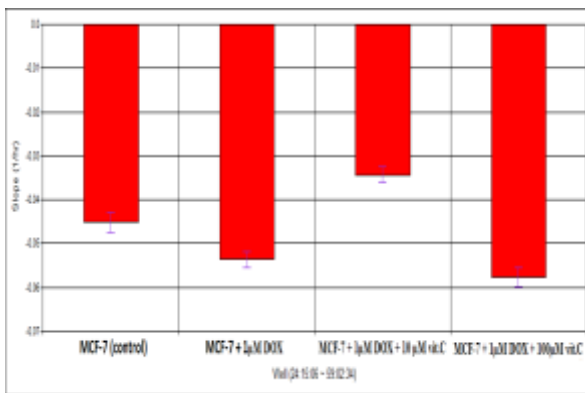


Figure 4: Curve slope of cell index values in time range: 24:15:06 ~ 59:02:34 during culturing MCF-7 cell line after the addition of cytotoxic drugs.

To examine the cytotoxic effect of different concentration vitamin C and 1 μ M doxorubicin, a standard chemotherapeutic agent, on MCF-7 cells, we seeded 20000 cells per well of E-Plate (Fig. 1). After 24 h and 15 min. MCF-7 cells were exposed to (10 μ M - 700 μ M) vitamin C together with 1 μ M doxorubicin. The higher the concentration of vitamin C applied together with the doxorubicin, the higher cytotoxic effect in MCF-7 cells is observed. The cell growth inhibition is thus dependent from the concentration and duration of exposure of the substance (Fig. 2A). Data are presented as a normalized cell index and normalized just after doxorubicin and different concentration of vitamin C treatment (CI; normalized at 24 h and 15 min.).

The normalized cell index values indicate the changes in MCF-7 proliferation/apoptosis after addition of DOX and DOX/vitamin C (Fig. 3). The lowest curve slope - 0.034 \pm 0.0018 h was observed for the MCF-7 cells with the

addition of DOX and 10 μ M vitamin C, whereas the highest of curve slope -0,058 \pm 0.0021 h for the MCF-7 cells with the addition of a 1 μ M doxorubicin and 100 μ M vitamin C (Fig. 4). This result confirmed the theory that at low vitamin C concentrations (<100 μ M vitamin C) the vitamin C acts as an antioxidant and decrease the effectivity of DOX treatment. However, at the higher concentrations (>100 μ M vitamin C) it examine the potential synergistic anticancer effect.

DISCUSSION

Although mammary cancer is the most common malignant neoplasia in women with a proportion of more than 458,000 deaths and 1,380,000 newly diagnosed cases each year, the mortality for this cancer has gradually decreased.¹⁵ In recent years the use of natural dietary antioxidants to minimize the cytotoxicity and the damage induced in normal tissues by antitumor agents is gaining consideration.¹⁶ One potential possible medical strategy is the use of vitamin C as adjunctive therapy with doxorubicin as the most effective chemotherapeutic drug applied in advanced mammary cancer therapy.² However, a major factor limiting use this drug is a cumulative, dose-dependent doxorubicin's cardiotoxicity.¹⁷ Two different mechanisms cardiotoxicity have been identified. The first implicates semiquinone-type free radicals produced in the NADPH-dependent reductase pathway. The second mechanism includes a non-enzymatic reaction of anthracyclines with iron that generates H_2O_2 .⁸ The resulting oxidative stress leads to cell damage, consequent on the alteration of enzymes, proteins, and DNA, and to lipid peroxidation, with the final formation of reactive electrophilic aldehydes.⁶ The following of these progressive development are cardiac dysfunction, cardiomyopathy, congestive heart failure and the apoptosis of cardiomyocytes.⁹

Vitamin C is considered to be one of the most potent and least toxic antioxidants for humans. As natural product is capable of blocking its reactive oxygen mediated cardiotoxicity effect and supported dose-dependent the activity traditional chemotherapeutic agent.⁶ It maintains the viability of cells during oxidative stress caused by the free oxygen radicals.^{17,15} Nowadays studies but come with findings that the higher doses parenteral administration of vitamin C act as a prooxidant. Experimental *in vitro* studies have shown potential of ascorbate to prevent the process of angiogenesis and metastatic spread by various anticancer mechanism.¹⁸ One possible mechanism is dependent on the redox status of the cell and its environment. If ascorbate is added to cells prior to oxidative stress, it functions as an antioxidant and so captures free radicals and affects the cell cycle at the G₂/M phase- allows DNA repair and reduces cytotoxicity and mutagenicity. Conversely, if its added during oxidative stress, it acts as a prooxidant.¹¹ As prooxidant does not readily react with oxygen to produce reactive oxygen species, but it readily donates an electron to redox-active transition metal ions (such as iron and

copper). These reduced metals can therefore react with oxygen to produce superoxide ions which, in turn, may dismutate to produce H_2O_2 .¹⁹ The hydrogen peroxide reduces cellular levels of thiols and can initiate membrane lipid peroxidation. Tumor cells are more sensitive to cytotoxic and mutagenic effects caused by oxidative stress via increased intracellular dehydroascorbic acid with following DNA damage.^{14,11}

The analysis different (controversial) effect as an antioxidant and a prooxidant of vitamin C was aim of this work. For comparison we used the study Ludke (2012_a) which deal with combination effect of vitamin C and doxorubicin by monitoring of cardiomyocytes. This publication shown that vitamin C was able to protect the cardiomyocytes from doxorubicine-induced necrosis. But, here is no adequate information about subcellular mechanisms of this beneficial effect. The most likely antioxidant effect is associated with a decrease in ASK-1 (apoptosis signal-regulating kinase 1) and p38 activation because these kinases are activated in various types of stress induced apoptosis including doxorubicin-induced cardiomyopathy. The most optimal concentration of vitamin C, in terms of viability of cardiac cells and free radicals production, was seen at 25 μM . The further increase to (50 μM and 100 μM) of vitamin C did not show any additional benefit. Despite of that this work has deal with combination of vitamin C and doxorubicin on cancer cells line, our result also shown that below concentrations at 10 μM vitamin C with 1 μM doxorubicin caused rather antioxidant effect (hypothetically cardioprotective action), but within anticancer combination therapy, decrease the efficacy of oncology treatment. The most significant antiproliferative effect on MCF-7 cells have been shown combination of vitamin C (700 μM) with doxorubicin (1 μM) (Fig. 2). Based on these findings we can follow controversial effect of vitamin C as unconventional anticancer agents with usually chemotherapeutic agent. If ascorbate is added to cardiac cells in dose 100 μM it functions as an antioxidant and decreased free radicals production. But if its added to cancer cells lines in the same dose (100 μM) it acts as a prooxidant and to produce the hydrogen peroxide with following DNA damage.

It is evident that the vitamin C treatment is dependent to a concentration at the specific time. The most important is also routes of administration. In patients suffering from cancer the low serum level of this vitamin has been proven (though corresponding to daily dosage) as it accumulates to a greater extent in tumor cells compared to normal tissue.²⁰ The oxidation of ascorbic first creates monodehydroascorbate followed by dehydroascorbic acid.^{21,22} The cancer cells readily take up ascorbate. Indeed, most tumors over express facilitative glucose transporters because of their high glycolytic metabolism which requires high glucose supply. As a consequence, dehydroascorbic acid can be transported by GLUTs (GLUT1), leading to the accumulation of vitamin C in tumors.¹³ According to routes of administration its

therefore mention that the effects of oral and parenteral administration of ascorbate are not comparable.^{23,14} The oral vitamin C dose, the more incompletely absorbed from the gut, probably as a result of a saturable transport mechanism. In addition, the dosing interval was too long with respect to the half-life of ascorbate. Overleaf, plasma ascorbate concentrations are often considerably higher soon after an intravenous dose than after oral administration.¹⁴ Thus only a high-doses parenteral administration of ascorbate are necessary to achieve beneficial results in cancer patients.²³ The intravenous doses can produce plasma concentrations 30- to 70-fold higher than the maximum tolerated oral doses.¹⁴ These findings also confirmed by this study. As shown figure 2A the higher concentration of vitamin C (700 μM) with doxorubicin has a higher cytotoxic effect in MCF-7 cells while the IC50 value of vitamin C was 493 μM (Fig. 2B).

CONCLUSION

Vitamin C exhibit diverse physiological roles as an antioxidant but also the surprising prooxidant activity. This work examined potential synergistic anticancer effect of doxorubicin with the vitamin C on human breast adenocarcinoma cell line in real time. This combination shown dose-dependent antiproliferative action, but only the higher concentration. The low doses potentially decrease chemotherapeutic effect. Due to the low toxicity of vitamin C even at very high concentrations, combinations ascorbic acid with doxorubicin, seem to be attractive for the future treatment of breast cancer with the possibility of reduction oncology side effects. However, it is important that further clinical trials will yield more clear information about possible mechanism action of vitamin C.

Acknowledgment: This work was supported by the Agency of the Slovak Ministry of Education, Science, Research and Sport of the Slovak Republic for the Structural Funds of EU, under project ITMS: 26220120067 and by VEGA 1/0103/16.

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Source of Support: Nil, Conflict of Interest: None.

